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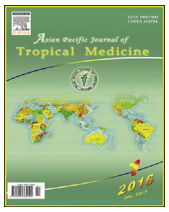

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Protective effect of hyperoside on cardiac ischemia reperfusion injury through inhibition of ER stress and activation of Nrf2 signaling

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ABSTRACT

Object: To study the protective effect of hyperoside (Hyp) on cardiac ischemia reperfusion injury and its potential mechanism.

Methods: Rats were divided into two groups for the evaluation, the Hyp (50 μM Hyp; $n = 8$) and the control group ($n = 8$). Rat hearts were isolated and perfused with Krebs-Henseleit buffer (KHB) for 30 min. After being inhibited with cardioplegic solution, they were stored for 4 h in B21 solution at 4 °C. Afterwards, rat hearts were perfused with KHB again for 45 min. In this period, Hyp was added into solutions of cardioplegia for storage and KHB. Parameters of cardiac functions, including heart rate, the systolic pressure of the left ventricle, the end-diastolic pressure of the left ventricle, the developed pressure of the left ventricle, the left-ventricular systolic pressure and the peak rise rate of the pressure of the left ventricle were recorded. The levels of adenosine triphosphate (ATP), the content of malondialdehyde and apoptotic cells were determined to evaluate the protective effect of Hyp on hearts suffered from ischemia reperfusion injury. Moreover, cultured cardiac myocytes were subjected to the process simulating ischemia/reperfusion. What were analyzed included the endoplasmic reticulum (ER) stress hallmarks expressions, such as binding immunoglobulin protein and C/EBP homologous protein, using the western blot and real-time PCR. Besides, the NF-E2-related factor 2 (Nrf2) expression was measured to explore the potential mechanism.

Results: Compared with the control group, the Hyp group had better cardiac functional parameters and higher ATP levels; pretreatment of Hyp greatly relieved the apoptosis of myocyte, decreased oxidative stress as well as ER stress and activated the signaling pathway of anti-oxidative Nrf2 to a further extent.

Conclusions: Hyp plays an important role in preserving cardiac function by improving ATP levels of tissue, easing oxidative injury of myocardium and reducing apoptosis following IRI dramatically, while the ER stress inhibition and the downstream Nrf2 signaling activation may contribute to the effects of protection.

1. Introduction

Percutaneous coronary intervention and coronary artery bypass grafting are indispensable as the most effective treatments for myocardial infarction [1,2]. During the period of ischemia, reperfusion injury occurs in the case of the insufficient protection of myocardium. A number of growth factors and cytokines are involved in the cardiac ischemia reperfusion injury (IRI) process, and cardiac IRI is multifactorial. Endoplasmic reticulum (ER) stress plays a key role in the process of IRI and apoptosis of myocytes [3,4]. The ER stress forms in a cell after the break of equilibrium in ER caused by unfolded/misfolded proteins accumulation, depletion of calcium and deprivation of energy during IRI. Unfolded protein response (UPR) is a process that a series of signaling pathways with conservative and complementary adaptive features are activated to deal with perturbations. It is also connected with classical translation control, such as decreased

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protein load and upregulated chaperones, including binding immunoglobulin protein (BiP) [5]. The apoptosis phase is initiated by the accumulation of C/EBP homologous protein (CHOP), phosphorylation of IRE1 and the activation of JNK, which leads to cell apoptosis in the end of IRI [6]. Both BiP and CHOP are generally regarded as the ER stress level hallmarks.

In UPR, the activation of NF-E2-related factor 2 (Nrf2) acts as a leading role. It has also been proven that Nrf2 is an essential transcriptional regulator considering genes coding detoxification enzymes expressions, proteins of antioxidant and other mediators of stress response [7]. It is reported by Liu *et al* that Nrf2-deficiency improves mice susceptibility to IRI and that Nrf2 is identified as a transcription factor of protection [8]. The UPR adaptive response includes various molecules and pathways for the stress condition compensation. A crosstalk exists between ER stress and Nrf2, as the former can activate the latter [9]. Regulating the axis of IRI-ER stress-Nrf2 presages has a significantly protective effect on IRI.

As a compound of flavonoid isolated from *Rhododendron ponticum* L., hyperoside (Hyp) is one of the primary components of traditional patent medicines of China, like capsules of Huang'kui, exerting anti-inflammatory properties [10], favorable effects of cardiovascular including anti-ischemic activities [11] and cytoprotective properties against oxidative stress through the elimination of reactive oxygen species and the improvement of the activity of antioxidant enzyme reported in our former study [12–14]. The exploration of additives with cardio-protective effects against IRI in solutions of cardioplegic is significant, as cardioplegic arrest protects the heart against ischemic injury. Notwithstanding, no data is available about the Hyp effects on cardiac functions as well as apoptosis after the prolonged conditions of cold ischemia. In this study we explored the protection of Hyp against the injury of reperfusion after the prolonged storage of hypothermia.

Before and/or after 4 h of global cold ischemia (4 °C), Hyp was included in both the preparation and the treatment of the isolated rat hearts in our study in order to explore whether there was reduced cardiac IRI by Hyp treatment during heart storage and perfusion. Experimental variables contained hemodynamic parameters, levels of adenosine triphosphate (ATP), content of malondialdehyde (MDA) and determination of cell apoptosis. Neonatal rat ventricular myocytes (NRVMCs) were cultured and subjected to simulated ischemia-reperfusion (sI/R) with or without the pretreatment of Hyp. The ER stress levels, activation of Nrf2 and apoptosis of the cell were measured *in vitro* for the purpose of disclosure the potential mechanism.

2. Materials and methods

2.1. Animal

Wistar rats [n = 16, weight: (220 ± 30) g] were obtained from Experimental Animal Center of Tongji University (Shanghai, China) and were evenly divided into two groups: the Hyp (n = 8) and the control group (n = 8). The treatment of all animals was based on the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the academy ethics committee of Tongji University. Hyp was purchased from Nanjing ZeLang Medical Technology Co., Ltd. (Nanjing, China).

2.2. Isolated heart preparation

Before the rapid excision and placement of rat hearts in cold (4 °C) Krebs-Henseleit buffer (KHB) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.0 mM glucose, pH 7.4, anesthetisation with intraperitoneal sodium pentobarbital (50 mg/kg) and intravenous injection with heparin (50 IU) were made to rats, as described previously [14]. Then the hearts were laid on a Langendorff apparatus (Radnoti, Monrovia, CA, USA) following the perfusion with gassed (95% O_2, 5% CO_2) KHB (37 °C) under the constant pressure of 80 mmHg. A small latex balloon filled with saline was inserted into the left atrium for the achievement of an end-diastolic pressure of 0–10 mmHg and then was pushed into the left ventricle through the mitral valve. The balloon was linked to a transducer of pressure (MP100; BIOPAC Systems, Goleta, CA, USA). BIOPAC MP150 (BIOPAC Systems) was used for digitization. AcqKnowledge 3.8.1 software (BIOPAC Systems) was used for the records of characteristic analog signals derived from the measurement of left ventricle pressure.

2.3. Protocols of perfusion

In the Hyp group, there was an addition of 50 μM Hyp into the perfusion fluid 10 min before IRI. The same protocol was obeyed by the two groups except the addition of Hyp to solutions of cardioplegia, to the storage solution during ischemia and to the solution of KHB during reperfusion process in the Hyp group.

2.4. Measurement of cardiac function

According to a previous study [15], the volume of the latex balloon was adjusted to achieve a left-ventricular end-diastolic pressure (LVEDP) of 0–10 mmHg during the stabilization phase. The maximal value of left-ventricular developed pressure (LVDP) increasing (+dp/dt) rates, the left-ventricular systolic pressure (LVSP), and LVEDP were recorded by a pressure transducer. The LVDP was calculated as LVDP = LVSP–LVEDP.

2.5. Measurement of myocardial ATP levels

ENLITEN® ATP Assay System (Promega Corp., Madison, WI, USA) was used for the quantification of ATP levels. In the final step of reperfusion, tissue specimens of myocardium were freezed in liquid nitrogen immediately and the individual pulverization was grinded into a fine powder by hand with a dry ice-chilled steel mortar and pestle [15]. Samples of myocardium (10 mg) were uniformized with 1 mL precooled extractant (0.1% trichloroacetic acid) and centrifuged at 680× g for 10 min. A 10-fold dilution was prepared on supernatant (100 μL) with 50 mmol/L Tris-acetate buffer including 2 mmol/L EDTA (pH 7.75). Afterwards, extract of 100 μL sample or solution of reference standard was put into a tube luminometer (Turner Designs Luminometer TD-20/20; Promega Corp.) before the auto-injection of 100 μL ATP luciferin/luciferase assay mix for the quantification of ATP. The measurement of luminescence was based on a set lag time of 1 s and a integration time of 10 s.

2.6. Measurement of myocardial MDA levels

In accordance with the kit specifications (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the instructions for manufacturers, the MDA content was detected with a visible-light photometer [15].

2.7. Cultured cardiac myocytes and sI/R

The isolation of rat hearts of 1-2-day-old Sprague–Dawley with the collagenase digestion and the passage purification through a Percoll gradient were conducted to obtain NRVMCs, as described [16]. NRVMCs were kept for 16 h in 2% FBS-supplemented medium, following the subjection to sI/R. For sI generally, glucose-free DMEM/F12 containing 2% dialyzed fetal bovine serum was used for the medium replacement. Then a gas-tight chamber with a BioSpherix PROOX model 110 controller was used to change the [O_2] to 0.1%. For sI/R after sI, the medium was replaced to DMEM/F12 containing glucose and 2% fetal bovine serum albumin and an incubator at 21% [O_2] was used for placing the cultures [17]. As for the treatment group, 50 μM Hyp was added 1 h before sI/R.

2.8. Determination of myocardial apoptotic cells

Assay kit of One Step TUNEL apoptosis assay kit was used for the determination of apoptotic cells. The technique of double-staining was carried out, apoptotic cell nuclei was quantified with the use of TUNEL staining (green fluorescence) and the total cell nuclei of myocardium was quantified with the use of DAPI staining (blue fluorescence), as described formerly [18]. Stained samples were observed by a fluorescence microscope (Olympus BX61, Japan) and five random visual fields were selected for each specimen.

2.9. Quantitative real-time polymerase chain reaction

In accordance with instructions of manufacturers, total RNA from cultured cells was extracted with TRIzol reagent (Invitrogen, Shanghai, China). cDNA was acquired from the transcription of total RNA (3–5 μg). The gene-specific primers for human *BiP*, *CHOP* and *β-actin* were showed as follows : *BiP*: forward: AAAGAAGACGGGCAAAGATGT, reverse: TGCT TGATGCTGAGAAGACAG. *CHOP* : forward: ACCACT CTTGACCCTGCTTCT, reverse: CTCTGGGAGGTGCTTGT-GAC. *β-actin*: forward: GTTGTCGACGACGAGCG, reverse: GCACAGAGCCTCGCCTT. With the combination of Eppendorf Mastercycler ep realplex system (Eppendorf, Hamburg, German) and the Absolute QPCR SYBR Green premix, a real-time chain reaction of quantitative polymerase was produced. The hot start for 15 min at 95 °C, the denaturation for 1 s at 95 °C, the annealing for 5 s at 60 °C and the extension for 10 s at 72 °C for 45 cycles constituted the amplification protocol. With the $2^{-\Delta\Delta Ct}$ method, expression levels were related to those of *β-actin* in the same samples for normalization.

2.10. Western blot

The harvest of NRVMC cells was followed by the twice washing with PBS. The extraction of extranuclear and intranuclear proteins were conducted in a separate way in accordance with the protein extraction kit protocol. The performance of western blot was previously described [18,19]. The detection of BiP, CHOP and Nrf2 was carried out with specific antibodies and expression analysis were performed after the normalization to references (β-actin in cytoplasm or Lamin B inside nucleus).

2.11. Statistical analysis

Experiments involving neonatal rat ventricular myocytes were carried out at least three times on different days (n = 5). Data were expressed as the mean ± standard error of the mean, and the data analysis was based on the SPSS (SPSS Inc. Chicago, IL). The variance analysis of Tukey's test was used for statistical analysis. A P-value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Cardiac function parameters

Cardiac function parameters were recorded and calculated by reference of Guo's study [15]. During the pre-ischemia (Table 1), no significant differences existed in functional parameters between the control group and the Hyp group. Parameters of cardiac functions, including LVSP, LVDP and +LV dp/dt_{max}, decreased largely in both the control and the Hyp groups in the period of reperfusion ($P < 0.05$; Table 1), demonstrating the destructive effect of I/R on the function of left ventricle. Rats in the Hyp group recovered better in the period of reperfusion following ischemia compared with rats in the control group. During reperfustion, parameters of functions in the Hyp group were tremendously higher compared with those of the control group ($P < 0.05$; Table 1). No indication of great differences in HR was observed (Table 1). Consequently, the results showed that the Hyp raised the heart tolerance to IRI.

3.2. Myocardial ATP and MDA levels

The result indicated that the ATP levels [(5.03 ± 0.20) μmol/g protein, n = 8] were greatly higher in the Hyp group compared with those of the control group [(1.22 ± 0.07) μmol/g protein, n = 8] ($P < 0.05$). The MDA content was lower in the Hyp group

Table 1
Variables in hemodynamics.

Variables	Control group	Hyp group
Pre-ischemia		
LVSP (mmHg)	143.7 ± 5.6	138.2 ± 5.9
LVEDP (mmHg)	13.3 ± 1.9	12.7 ± 1.8
LVDP (mmHg)	130.4 ± 5.1	125.5 ± 5.0
+LV dp/dt_{max} (mmHg/s)	4 723 ± 228	4 933 ± 266
HR (beats/min)	230.5 ± 23.2	232.8 ± 23.0
Reperfusion		
LVSP (mmHg)	104.4 ± 5.1	121.3 ± 5.8
LVEDP (mmHg)	70.5 ± 6.8[a]	35.2 ± 5.3[a,b]
LVDP (mmHg)	33.9 ± 2.7[a]	86.1 ± 2.6[a,b]
+LV dp/dt_{max} (mmHg/s)	1 180 ± 103[a]	3 110 ± 157[a,b]
HR (beats/min)	225.7 ± 35.2	229.5 ± 47.1

[a]$P < 0.05$, *vs* respective pre-ischemia value; [b]$P < 0.05$, *vs* control group. +LV dp/dt_{max}, peak rise rate of the pressure of the left ventricle; HR, heart rate. Data were expressed as the mean ± SE.

[(1.96 ± 0.09) μmol/g protein, n = 8] than that in the control group [(4.32 ± 0.19) μmol/g protein, n = 8] ($P < 0.05$).

3.3. Myocytes apoptosis stimulated by I/R

Cultured NRVMCs of the I/R group were treated with sI/R and NRVMCs of the control group were free from treatment. As for the I/R+Hyp group, 50 μM Hyp was added 1 h before sI/R. Compared with the I/R group, percentage of TUNEL-positive staining nuclei in NRVMCs of the I/R+Hyp group (68.7% ± 6.5% *vs* 10.1% ± 3.8%, n = 5, $P < 0.001$) significantly decreased after sI/R. Hyp administration greatly suppressed apoptosis of myocytes in the I/R+Hyp group than that in the I/R group.

3.4. Hyp reduced ER stress induced by I/R

The assessment of ER stress could be generally made by the BiP and CHOP expression. Hyp pretreatment was observed to down-regulate the mRNA and protein levels of BiP and CHOP compared with the control group, which indicated that Hyp reduced ER stress in NRVMC myocytes caused by sI/R (Table 2).

Table 2
BiP and CHOP expression.

Relative expression (related to β-actin)	Control group	I/R group	I/R+Hyp group
BiP			
mRNA($2^{-\Delta\Delta Ct}$)	3.6 ± 0.9	23.7 ± 3.8	17.7 ± 1.5[a]
Protein	0.11 ± 0.01	0.89 ± 0.04	0.52 ± 0.03[b]
CHOP			
mRNA($2^{-\Delta\Delta Ct}$)	4.4 ± 0.8	18.3 ± 3.5	12.8 ± 2.7[a]
Protein	0.12 ± 0.01	0.76 ± 0.05	0.48 ± 0.01[b]

Data were expressed as the mean ± SE.
[a]$P < 0.05$, *vs* I/R group; [b]$P < 0.01$, *vs* I/R group. n = 5.

3.5. Hyp promoted the expression of Nrf2 induced by I/R

I/R up-regulated both the expression of extranuclear and intranuclear Nrf2. Pretreatment of Hyp increased the expression of antioxidant transcription factor Nrf2, indicating that the UPR adaptive response was activated by this treatment in a further way (Table 3).

Table 3
Nrf2 expression.

Nrf2	Control group	I/R group	I/R+Hyp group
Intranuclear expression	0.20 ± 0.01	0.62 ± 0.08	0.85 ± 0.10[a]
Extranuclear expression	0.19 ± 0.02	0.36 ± 0.04	0.61 ± 0.05[a]

Data were expressed as the mean ± SE.
[a]$P < 0.01$, *vs* I/R group. n = 5. Intranuclear expression: related to Lamin B; extranuclear expression: related to β-actin.

4. Discussion

It has been shown that Hyp, which is a major compound of flavonoid isolated from *Rhododendron ponticum* L., has protective effects on kidney against chronic fibrotic disease by inhibition of apoptosis and inflammation [13]. In this study, Hyp was used as a protective agent of myocardium and showed its therapeutic effects on the promotion of cardiac function and the apoptosis inhibition. It was a creative study for the analysis of Hyp effects on a cardiac model of 4-h cold cardioplegia and reperfusion to mimic clinical IRI of heart in the operation of coronary artery bypass grafting.

Hyp owns numerous biological effects as the fundamental ingredient of some medicines, like capsules of Huang'kui, including its activities in fighting against inflammation, virus, oxidant and cancer [20,21]. Furthermore, Hyp has favorable cardiovascular effects, for instance, activities of anti-ischemia [11]. Studies have supported the anti-apoptosis effect of Hyp in cells of V79-4 and PC12 [22]. In line with the protective effects of Hyp which were stated previously, our study revealed that Hyp greatly promoted heart function, like LVDP and +LV dp/dt_{max} in an IRI model.

ROS was firstly involved in the protective mechanism of Hyp. It was indicated in a previous study that Hyp cleaned the hydroxyl radical (·OH) and the effects depended on dose [23]. As ·OH is regarded as the most cytotoxic radical of free oxygen, these consequences illustrated that a better recovery of cardiac function could be achieved by Hyp administration in the storage and in the perfusion after the cold global ischemia. ROS at reflow after the ischemia may increase peroxidation of mitochondrial membranes and activities of metabolic enzyme, then inhibited heart metabolic recovery and functional parameters. The recovery of phosphate contents of high-energy could be enhanced at the reflow when radical toxicity of free oxygen falled due to free radical scavengers, which showed a connection between radical production of free oxygen as well as the impairment of energy metabolism of myocardium in the period of reperfusion [24]. Here lipid peroxidation decrease was shown in the Hyp group, indicating that the free radical injury reduced in the Hyp group compared with the control group.

Myocytes apoptotic inhibition was considered as the second mechanism. In our study, the Hyp group showed significantly less cell apoptosis compared with the control, indicating that Hyp protected myocytes from cytotoxicity caused by IRI. What were discussed here included ER stress and one of its primary downstream factors, Nrf2, for the investigation of the potential mechanism. BiP is a significant chaperone of ER lumen, and it has an interaction with polypeptide folding to control the maturation of nascent glycoprotein structures [25]. Moreover, as a stress protein, BiP expression level is intimately connected with the ER stress intensity [26]. Another hallmark associated with intensity of ER stress is CHOP [27]. It has been shown that CHOP is a significant factor in the period of apoptosis induced by ER stress. When CHOP is deleted, apoptosis may be reduced. When CHOP is overexpressed, the cell apoptosis may be increased [6,28]. It was shown that the treatment of Hyp could reduce both BiP and CHOP expressions significantly, which indicated a decreased ER stress. The CHOP down-regulation was considered as a possible mechanism of the inhibition of myocyte apoptosis. When an intensive ER stress was improved, UPR could change from the phase of apoptosis to adaptive responses [29]. Nrf2, a transcription factor induced by antioxidant response, could be activated by UPR machinery adaptive responses as well. As literature reported, based on the ER stress context, the Nrf2 activator could be

taken as PERK and the axis of IRE1α-JNK-Nrf2 seemed to affect the Nrf2 activation [30]. Our experiments further revealed that both the intracellular and extracellular levels of Nrf2 were up-regulated, suggesting that Nrf2 was significantly activated by the treatment of Hyp.

To sum up, Hyp could preserve cardiac functions in a great way, including enhancement of ATP levels, reduction of myocardial oxidative injury and myocyte apoptosis. Furthermore, the potential mechanism is associated with ER stress inhibition as well as the activation of Nrf2 signaling. Notwithstanding, studies should be added one step ahead. For expanding the clinical utility spectrum, evaluation of Hyp protection capacity on myocardium in other models of cardiac ischemia should be made in the investigations to a greater degree. Moreover, the question of whether higher Hyp doses bring greater protective effects on myocardium should be answered in future studies.

Conflicts of interest statement

We declare that we have no conflict of interest.

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